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FORM PTO-1390 (REV 11-98)		U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE		ATTORNEY'S DOCKET NUMBER	
TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371				14538A-004010US	
				U.S. APPLICATION NO. (If known, see 37 CFR 1.5) 09/486293	
INTERNATIONAL APPLICATION NO. PCT/US98/17384		INTERNATIONAL FILING DATE 21 August 1998		PRIORITY DATE CLAIMED 21 August 1997	
TITLE OF INVENTION ISOLATION AND EXPRESSION OF A DISABLED PROTEIN GENE MdaB1 AND METHODS					
APPLICANT(S) FOR DO/EO/US Jonathan A. Cooper and Brian W. Howell					
Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information.					
<ol style="list-style-type: none"> 1. <input checked="" type="checkbox"/> This is a FIRST submission of items concerning a filing under 35 U.S.C. 371. 2. <input type="checkbox"/> This is a SECOND or SUBSEQUENT submission of items concerning a filing under 35 U.S.C. 371. 3. <input checked="" type="checkbox"/> This express request to begin national examination procedures (35 U.S.C. 371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. 371(b) and PCT Articles 22 and 39(1). 4. <input checked="" type="checkbox"/> A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date. 5. <input checked="" type="checkbox"/> A copy of the International Application as filed (35 U.S.C. 371(c)(2)) <ol style="list-style-type: none"> a. <input checked="" type="checkbox"/> is transmitted herewith (required only if not transmitted by the International Bureau). b. <input type="checkbox"/> has been transmitted by the International Bureau. c. <input type="checkbox"/> is not required, as the application was filed in the United States Receiving Office (RO/US). 6. <input type="checkbox"/> A translation of the International Application into English (35 U.S.C. 371(c)(2)). 7. <input type="checkbox"/> Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3)) <ol style="list-style-type: none"> a. <input type="checkbox"/> are transmitted herewith (required only if not transmitted by the International Bureau). b. <input type="checkbox"/> have been transmitted by the International Bureau. c. <input type="checkbox"/> have not been made; however, the time limit for making such amendments has NOT expired. d. <input checked="" type="checkbox"/> have not been made and will not be made. 8. <input type="checkbox"/> A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)). 9. <input type="checkbox"/> An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)). 10. <input type="checkbox"/> A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)). 					
Items 11. to 16. below concern document(s) or information included:					
11. <input type="checkbox"/> An Information Disclosure Statement under 37 CFR 1.97 and 1.98.					
12. <input type="checkbox"/> An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.					
13. <input type="checkbox"/> A FIRST preliminary amendment.					
<input type="checkbox"/> A SECOND or SUBSEQUENT preliminary amendment.					
14. <input type="checkbox"/> A substitute specification.					
15. <input type="checkbox"/> A change of power of attorney and/or address letter.					
16. <input type="checkbox"/> Other items or information:					

U.S. APPLICATION NO. (R. E. No. 1) (CFR 1.51)		INTERNATIONAL APPLICATION NO. PCT/US98/17384		ATTORNEY'S DOCKET NUMBER 14538A-004010US																					
17. <input checked="" type="checkbox"/> The following fees are submitted: BASIC NATIONAL FEE (37 CFR 1.492 (a) (1) - (5)) : Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO. \$970.00 International preliminary examination fee (37 CFR 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO \$840.00 International preliminary examination fee (37 CFR 1.482) not paid to USPTO but international search fee (37 CFR 1.445(a)(2)) paid to USPTO \$760.00 International preliminary examination fee paid to USPTO (37 CFR 1.482) but all claims did not satisfy provisions of PCT Article 33(1)-(4) \$670.00 International preliminary examination fee paid to USPTO (37 CFR 1.482) and all claims satisfied provisions of PCT Article 33(1)-(4) \$96.00 ENTER APPROPRIATE BASIC FEE AMOUNT =				CALCULATIONS PTO USE ONLY																					
Surcharge of \$130.00 for furnishing the oath or declaration later than <input type="checkbox"/> 20 <input checked="" type="checkbox"/> 30 months from the earliest claimed priority date (37 CFR 1.492(e)).				\$ 670																					
<table border="1" style="width: 100%; border-collapse: collapse;"> <thead> <tr> <th style="width: 20%;">CLAIMS</th> <th style="width: 20%;">NUMBER FILED</th> <th style="width: 20%;">NUMBER EXTRA</th> <th style="width: 20%;">RATE</th> </tr> </thead> <tbody> <tr> <td>Total claims</td> <td>35 - 20 =</td> <td>15</td> <td>X \$18.00</td> </tr> <tr> <td>Independent claims</td> <td>8 - 3 =</td> <td>5</td> <td>X \$78.00</td> </tr> <tr> <td colspan="3">MULTIPLE DEPENDENT CLAIM(S) (if applicable)</td> <td>+ \$260.00</td> </tr> <tr> <td colspan="3">TOTAL OF ABOVE CALCULATIONS =</td> <td></td> </tr> </tbody> </table>				CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE	Total claims	35 - 20 =	15	X \$18.00	Independent claims	8 - 3 =	5	X \$78.00	MULTIPLE DEPENDENT CLAIM(S) (if applicable)			+ \$260.00	TOTAL OF ABOVE CALCULATIONS =				\$ 130	
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Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). \$40.00 per property				\$																					
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a. <input type="checkbox"/> A check in the amount of \$ _____ to cover the above fees is enclosed. b. <input checked="" type="checkbox"/> Please charge my Deposit Account No. <u>20-1430</u> in the amount of \$ <u>1,460</u> to cover the above fees. A duplicate copy of this sheet is enclosed. c. <input checked="" type="checkbox"/> The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. <u>20-1430</u> . A duplicate copy of this sheet is enclosed.																									
NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.																									
SEND ALL CORRESPONDENCE TO. Brian Poor Townsend and Townsend and Crew 2 Embarcadero Center, 8th Floor San Francisco, CA 94111																									
<div style="display: flex; justify-content: space-between; align-items: flex-end;"> <div style="width: 40%;"> SIGNATURE. </div> <div style="width: 50%;"> <u>Brian W. Poor</u> NAME 32,928 REGISTRATION NUMBER </div> </div>																									

ISOLATION AND EXPRESSION OF A DISABLED PROTEIN GENE
mDab1 AND METHODS

GOVERNMENT SUPPORT

This work was supported by grants CA41097, CA41072, HD25326 and HD24875 from the National Institutes of Health. The U.S. Government may have certain rights in the invention.

RELATED APPLICATIONS

The present application claims the benefit and is a continuation-in-part application of U.S. Provisional Serial No. 60/056,473, filed August 21, 1997, which is incorporated herein by reference.

BACKGROUND OF THE INVENTION

Numerous developmental processes are regulated by signaling cascades that alter protein phosphotyrosine levels. Many extracellular cues are linked to cellular responses via transmembrane receptor protein-tyrosine kinases (PTKs) and phosphatases (PTPs). In the nervous system, transmembrane kinases and phosphatases are required for neuronal differentiation and survival, neurite extension, the directed growth of the neuronal growth cone, and the fasciculation of nerve bundles (Snider, Cell 77:627-638 (1994); Callahan et al., Nature 376:171-174 (1995); Tessier-Lavigne, Cell 82:345-348 (1995); Desai et al., Cell 84:599-609 (1996); Krueger et al., Cell 84:611-622 (1996)). These transmembrane receptors are directly regulated by specific ligands. Cytoplasmic PTKs are also involved in the development of the nervous system, although the ligands which induce their activation are less well understood (Gertler et al., Cell 58:103-113 (1989); Grant et al., Science 258:1903-1910 (1992); Umemori et al., Nature 367:572-576 (1994)). There is growing evidence that these kinases are regulated in pathways responding to components of the extracellular milieu and may function to regulate axonal growth downstream of receptors that lack intrinsic kinase

activity (Bixby & Harris, Ann. Rev. Cell. Biol. 7:117-159 (1991)).

The non-receptor PTK Src is highly expressed in the developing mammalian nervous system (Maness et al., Adv. Exp. Med. & Biol. 265:117-125, (1990); Maness, Dev. Neurosci. 14:257-270 (1992)). During neurogenesis Src kinase activity increases and Src becomes concentrated in growth cones of neurons. Growth cones migrate by extending actin-rich filopodia and lamellipodia, and tyrosine phosphorylation is important for the formation of these actin structures (Wu & Goldberg, J. Cell Biol. 123:653-664 1993; Goldberg & Wu, J. Neurobiol. 27:553-560 (1995)). Neurons cultured from mice that lack Src extend neurites less well than wild-type neurons when plated on surfaces coated with the neural cell adhesion molecule L1 (Ignelzi et al., Neuron 12:873-884 (1994)). This defect is specific, since neurons lacking the Src relatives Fyn or Yes extend neurites normally (Beggs et al., J. Cell Biol. 127:825-833 (1994)). Moreover, neurons from mice lacking Fyn extend only short neurites on NCAM-140 but extend long neurites on L1 (Beggs et al., *ibid.*). Src and Yes are not needed for neurite extension on NCAM-140. These specific defects point to the existence of adhesion-stimulated, Src- and Fyn-dependent, regulatory processes required for neurite extension. Signals from neurotrophin receptor PTKs, such as TrkA, may also be relayed through Src. Nerve growth factor- (NGF) induced neurite extension is Src dependent in PC12 pheochromocytoma cells (Kremer et al., J. Cell Biol. 115:809-819 (1991); Vaillancourt et al., Mol. Cell. Biol. 15:3644-3653 (1995)).

The non-receptor tyrosine kinase, Abl, participates in nervous system development in *Drosophila*. The *Drosophila* Abl (dAbl) protein is found in many cell types in the developing embryo, but expression is highest in the cell bodies and axons of neurons in the developing central nervous system (CNS) (Gertler et al., *ibid.* (1989); Bennett & Hoffmann, Devel. 116:953-966 (1992)). Flies lacking the dAbl gene develop past metamorphosis but die as adults before or soon after eclosion (Henkemeyer et al., Cell 51:821-828

(1987)). Five genes were identified in screens for dominant second site mutations that exacerbate the *dAbl*⁻ phenotype and have been dubbed HDA (haploinsufficient, dependent upon *dAbl*) genes (Gertler et al., *ibid.*, (1989); Hill et al., Genetics 141:595-606 (1995)). When heterozygous for a mutation in a HDA gene, *dAbl*⁻ but not *dAbl*⁺ embryos die as embryos, with a characteristic terminal phenotype. The neurons of the CNS are present in normal number and extend axons, but gaps are apparent in the commissural and longitudinal axon bundles (Gertler et al., *ibid.* (1989), Gertler et al., Genes Dev. 7:441-453 (1993); Hill et al., *ibid.* (1995)). Three of the HDA genes, *disabled* (*dab*), *prospero*, and *fax* have been cloned and have distinct properties (Vaessin et al., Cell 67:941-953 (1991); Gertler et al., *ibid.* (1993); Hill et al., *ibid.* (1995)). Homozygous mutations in the HDA genes *dab* and *fax* in a *dAbl* mutant background results in almost complete loss of CNS axonal tracts. The *dab* and *fax* genes also show dosage sensitive interactions with each other (Gertler, "Genetic Modifiers of the *Drosophila abl* mutant phenotype," Ph.D. Dissertation, University of Wisconsin-Madison (1992); Hill et al., *ibid.* (1995)) as well as with *dAbl* and therefore may have related functions.

The *Drosophila dab* gene encodes a 2412 residue protein (Dab) that co-localizes with *dAbl* to the cell bodies and axons of embryonic CNS neurons (Gertler et al., *ibid.* (1993)). In *Drosophila* Dab is essential for normal CNS development, even in the presence of *dAbl*. Dab is tyrosine phosphorylated in insect cells and, given the co-localization with *dAbl* in the CNS, it has been suggested that Dab may be a physiological substrate of *dAbl* (Gertler et al., *ibid.* (1993)). However, the role of tyrosine phosphorylation in regulating Dab function, and the identities of the PTKs that phosphorylate Dab, remain unclear. The kinase activity of *dAbl* is dispensable for normal embryonic development, unless the levels of Dab or other HDA gene products are reduced by heterozygous mutations (Henkemeyer et al., Cell 63:949-960 (1990)). Using a temperature-sensitive mutant, *dAbl* kinase activity was shown to be required in *dab* heterozygotes after

the time of cell fate specification and during the time of axonogenesis in the embryonic CNS (Henkemeyer et al., *ibid.* (1990)). Despite the loss of nerve bundles in the CNS, the total number of neurons is unaffected (Gertler et al., *Science* 248:857-860 (1990)). These results suggest that dAbl has kinase dependent and independent roles in development. Other PTKs that are expressed in the fly CNS, such as *Drosophila* Src(dSrc) (Simon et al., *Cell* 42:831-840 (1985)), may substitute for the dAbl kinase requirement in CNS development, provided the levels of Dab are normal.

SUMMARY OF THE INVENTION

mDab1, a mammalian homolog of Dab, is identified. mDab1 was cloned based upon its interaction with Src in a yeast two-hybrid screen. The *mdab1* gene is expressed as a variety of spliced mRNAs in the nervous system and in some cell lines, and mDab1 proteins are differentially expressed and tyrosine phosphorylated during neural development. When phosphorylated on tyrosine, mDab1 binds to the SH2 domains of Src, Fyn and Abl. mDab1 also forms complexes with cellular phosphotyrosyl proteins through a phosphotyrosine-binding (PTB) domain. mDab1 appears to play a role as an adaptor protein that participates in development of the nervous system.

The present invention further demonstrates that disruption of the *mammalian disabled1* (*mdab1*) gene disturbs neuronal layering in the cerebral cortex, hippocampus and cerebellum.

In further aspects of the invention, mDab1 proteins, peptides, fusion proteins and antibodies are used in a variety of screening and diagnostic methods.

DESCRIPTION OF THE SPECIFIC EMBODIMENTS

The present invention identifies a mammalian homolog of the *Drosophila* Disabled (Dab) protein, mDab1, and shows it is an adaptor molecule functioning in neural development.

More specifically, the present invention provides representative nucleotide sequences encoding murine Dab1.

It is an object of the present invention to provide representative polynucleotide molecules and amino acids sequences encoding mDab1. Sequences encoding mDab1 include those sequences that are identical or result in minor variations in amino acid sequence, such as those due to genetic polymorphisms, differences between species and those in which blocks of amino acids have been added, altered or replaced without substantially altering the biological activity of the proteins.

The terms "identical" or percent "identity," in the context of two or more nucleic acids or polypeptide sequences, refer to two or more sequences or subsequences that are the same or have a specified percentage of amino acid residues or nucleotides that are the same, when compared and aligned for maximum correspondence, as measured using one of the following sequence comparison algorithms or by visual inspection.

The phrase "substantially identical," in the context of two nucleic acids or polypeptides, refers to two or more sequences or subsequences that have at least 60%, preferably 80%, most preferably 90-95% nucleotide or amino acid residue identity, when compared and aligned for maximum correspondence, as measured using one of the following sequence comparison algorithms or by visual inspection. Preferably, the substantial identity exists over a region of the sequences that is at least about 50 residues in length, more preferably over a region of at least about 100 residues, and most preferably the sequences are substantially identical over at least about 150 residues. In a most preferred embodiment, the sequences are substantially identical over the entire length of the coding regions.

For sequence comparison, typically one sequence acts as a reference sequence, to which test sequences are compared. When using a sequence comparison algorithm, test and reference sequences are input into a computer, subsequence coordinates are designated, if necessary, and sequence algorithm program parameters are designated. The sequence comparison algorithm

then calculates the percent sequence identity for the test sequence(s) relative to the reference sequence, based on the designated program parameters.

Optimal alignment of sequences for comparison can be conducted, e.g., by the local homology algorithm of Smith & Waterman, Adv. Appl. Math. 2:482 (1981), by the homology alignment algorithm of Needleman & Wunsch, J. Mol. Biol. 48:443 (1970), by the search for similarity method of Pearson & Lipman, Proc. Natl. Acad. Sci. USA 85:2444 (1988), by computerized implementations of these algorithms (GAP, BESTFIT, FASTA, and TFASTA in the Wisconsin Genetics Software Package, Genetics Computer Group, 575 Science Dr., Madison, WI), or by visual inspection (see generally Ausubel et al., *supra*).

One example of a useful algorithm is PILEUP. PILEUP creates a multiple sequence alignment from a group of related sequences using progressive, pairwise alignments to show relationship and percent sequence identity. It also plots a tree or dendrogram showing the clustering relationships used to create the alignment. PILEUP uses a simplification of the progressive alignment method of Feng & Doolittle, J. Mol. Evol. 35:351-360 (1987). The method used is similar to the method described by Higgins & Sharp, CABIOS 5:151-153 (1989). The program can align up to 300 sequences, each of a maximum length of 5,000 nucleotides or amino acids. The multiple alignment procedure begins with the pairwise alignment of the two most similar sequences, producing a cluster of two aligned sequences. This cluster is then aligned to the next most related sequence or cluster of aligned sequences. Two clusters of sequences are aligned by a simple extension of the pairwise alignment of two individual sequences. The final alignment is achieved by a series of progressive, pairwise alignments. The program is run by designating specific sequences and their amino acid or nucleotide coordinates for regions of sequence comparison and by designating the program parameters. For example, a reference sequence can be compared to other test sequences to determine the percent sequence identity relationship using the following parameters: default

Another example of algorithm that is suitable for determining percent sequence identity and sequence similarity is the BLAST algorithm, which is described in Altschul et al., J. Mol. Biol. 215:403-410 (1990). Software for performing BLAST analyses is publicly available through the National Center for Biotechnology Information (<http://www.ncbi.nlm.nih.gov/>). This algorithm involves first identifying high scoring sequence pairs (HSPs) by identifying short words of length W in the query sequence, which either match or satisfy some positive-valued threshold score T when aligned with a word of the same length in a database sequence. T is referred to as the neighborhood word score threshold (Altschul et al., supra). These initial neighborhood word hits act as seeds for initiating searches to find longer HSPs containing them. The word hits are then extended in both directions along each sequence for as far as the cumulative alignment score can be increased. Cumulative scores are calculated using, for nucleotide sequences, the parameters M (reward score for a pair of matching residues; always > 0) and N (penalty score for mismatching residues; always < 0). For amino acid sequences, a scoring matrix is used to calculate the cumulative score. Extension of the word hits in each direction are halted when: the cumulative alignment score falls off by the quantity X from its maximum achieved value; the cumulative score goes to zero or below, due to the accumulation of one or more negative-scoring residue alignments; or the end of either sequence is reached. The BLAST algorithm parameters W, T, and X determine the sensitivity and speed of the alignment. The BLASTN program (for nucleotide sequences) uses as defaults a wordlength (W) of 11, an expectation (E) of 10, M=5, N=-4, and a comparison of both strands. For amino acid sequences, the BLASTP program uses as defaults a wordlength (W) of 3, an expectation (E) of 10, and the BLOSUM62 scoring matrix (see Henikoff & Henikoff, Proc. Natl. Acad. Sci. USA 89:10915 (1989)).

mDab1 of the present invention has been shown to comprise a phosphotyrosine binding domain and has been shown to be capable of binding to/or associating with SH2 domains of Src, Fyn and Abl. Further, the disclosed polynucleotide sequences or portions thereof can be used to identify and isolate mammalian Dab1 polynucleotide molecules from suitable hosts such as canine, ovine, bovine, caprine, lagomorph or the like. In particular, the nucleotide sequences encoding the phosphotyrosine binding domain can be used to identify polynucleotide molecules encoding mDab1. Complementary DNA molecules encoding mDab1 may be obtained by constructing a cDNA library mRNA from, for example, brain. DNA molecules encoding mDab1 may be isolated from such a library using the disclosed sequences in standard hybridization techniques

The choice of hybridization conditions will generally be guided by the purpose of the hybridization, the type of hybridization (DNA-DNA or DNA-RNA), and the level of relatedness between the sequences. Methods for hybridization are well established in the literature; See, for example: Sambrook, *ibid.*; Hames and Higgins, eds, Nucleic Acid Hybridization A Practical Approach, IRL Press, Washington DC, 1985; Berger and Kimmel, eds, Methods in Enzymology, Vol. 52, Guide to Molecular Cloning Techniques, Academic Press Inc., New York, NY, 1987; and Bothwell, Yancopoulos and Alt, eds, Methods for Cloning and Analysis of Eukaryotic Genes, Jones and Bartlett Publishers, Boston, MA 1990; which are incorporated by reference herein in their entirety. The stability of nucleic acid duplexes will decrease with an increased number and location of mismatched bases; thus, the stringency of hybridization may be used to maximize or

In another method for isolating mDab related polynucleotide sequences, a modified yeast two-hybrid system can be used. This method provides that a library of cDNA from a cellular source is prepared and inserted into an expression that permits the expression of the inserted cDNA. A particularly preferred cellular source is brain or neuronal tissue. The prepared cDNA library is screened for binding to expressed Src proteins. For example, Src-Lex A fusion constructs can be prepared from wild type Src DNA. Clones containing inserts demonstrated to express proteins capable of binding to the Src containing fusion proteins are selected and the inserts isolated and analyzed for relatedness to the disclosed mDab1 polynucleotide sequences as disclosed herein.

The invention also provides isolated and purified polynucleotide molecules encoding mDab1 capable of hybridizing under stringent conditions to an oligonucleotide of 15 or more

The present invention also provide methods for producing recombinant mDab1 by inserting a DNA molecule encoding mDab1 into a suitable expression vector, which is in turn used to transfect or transform a suitable host cell. Suitable expression vectors for use in carrying out the present invention will generally comprise a promoter capable of directing the transcription of a polynucleotide molecule of interest in a host cell. Representative expression vectors may include both plasmid and/or viral vector sequences. Suitable vectors include retroviral vectors, vaccinia viral vectors, CMV viral vectors, BLUESCRIPT, baculovirus vectors, and the like. Promoters capable of directing the transcription of a cloned gene or cDNA may be inducible or constitutive promoters and include viral and cellular promoters. For expression in mammalian host cells, suitable viral promoters include the immediate early cytomegalovirus promoter (Boshart et al., Cell 41: 521-530, 1985) and the SV40 promoter (Subramani et al., Mol. Cell. Biol. 1: 854-864, 1981). Suitable cellular promoters for expression of proteins in mammalian host cells include but are not limited to the mouse metallothionien-1 promoter (Palmiter et al., U.S. Patent No. 4,579,821), and tetracycline-responsive promoter (Gossen and Bujard, Proc. Natl. Acad. Sci.USA 89: 5547-5551, 1992 and Pescini et al., Biochem. Biophys. Res. Comm. 202: 1664-1667, 1994). Also contained in the expression vectors is a transcription termination signal located downstream of the coding sequence of interest. Suitable transcription termination signals include the early or late polyadenylation signals from SV40 (Kaufman and Sharp, Mol. Cell. Biol. 2:1304-1319, 1982), the polyadenylation signal from the Adenovirus 5

e1B region and the human growth hormone gene terminator (DeNoto et al., Nucleic Acid. Res. 9: 3719-3730, 1981).

Mammalian cells may be transfected by a number of methods including calcium phosphate precipitation (Wigler et al., Cell 14: 725, 1978; Corsaro and Pearson, Somatic Cell Genetics 7: 603, 1981; Graham and Van der Eb, Virology 52: 456, 1973); lipofection (Felgner et al., Proc. Natl. Acad. Sci. USA 84: 7413-7417, 1987), microinjection and electroporation (Neumann et al., EMBO J. 1: 8410845, 1982). Mammalian cells can be transduced with virus such as SV40, CMV and the like. In the case of viral vectors, cloned DNA molecules may be introduced by infection of susceptible cells with viral particles. Retroviral vectors may be preferred for use in expressing mDab1 in mammalian cells.

It may be preferable to use a selectable marker to identify cells that contain the cloned DNA. Selectable markers are generally introduced into the cells along with the cloned DNA molecules and include genes that confer resistance to drugs, such as neomycin, hygromycin and methotrexate. Selectable markers may also complement auxotrophies in the host cell. Yet other selectable markers provide detectable signals, such as beta-galactosidase to identify cells containing the cloned DNA molecules. Selectable markers may be amplifiable. Such amplifiable selectable markers may be used to amplify the number of sequences integrated into the host genome.

As would be evident to one of ordinary skill in the art, the polynucleotide molecules of the present invention may be expressed *Saccharomyces cerevisiae*, filamentous fungi, and *E. coli*. Methods for expressing cloned genes in *Saccharomyces cerevisiae* are generally known in the art (see, "Gene Expression Technology," Methods in Enzymology, Vol. 185, Goeddel (ed.), Academic Press, San Diego, CA, 1990 and "Guide to Yeast Genetics and Molecular Biology," Methods in Enzymology, Guthrie and Fink (eds.), Academic Press, San Diego, CA, 1991; which are incorporated herein by reference). Filamentous fungi (e.g., strains of *Aspergillus*) may also be used to express the proteins of the present invention.

Tyrosine phosphorylated mDab1 associates with the SH2 domains of Src, Fyn and Abl. As disclosed in more detail herein, mDab1 and Src interact when P19 embryonal carcinoma

mDab1 can form complexes with cellular phosphotyrosyl proteins through a domain that is related to the PTB domains of the Shc family of adaptor proteins. The importance of the mDab1 PTB is suggested by its conservation across the Dab family, but it is sufficiently divergent in sequence from the Shc family of PTBs to question whether it adopts a similar structure. PTBs are difficult to recognize by primary sequence alone. The IRS-1 PTB, for example, is highly divergent in primary sequence, yet is fully functional and has a similar structure to the ShcA PTB (Zhou et al., *ibid.* (1996)). PTBs can bind to phosphotyrosyl peptides and to polyphosphoinositides (Zhou et al., *ibid.* (1995)). Peptides containing the Asn Pro Xaa pTyr (NPXpY) consensus sequence bind to the ShcA. However, some PTB domains have been discovered that can bind non-phosphorylated proteins. The brain proteins FE65 and X11, for example, have PTB domains that bind a non-phosphorylated sequence, Asn Pro Xaa Tyr present in the amyloid precursor protein. The molecular structure of the X11 PTB domain with the non-phosphorylated peptide bound shows many similarities with the Shc and IRS-1 PTB domains. The PTB domain of Numb was shown to bind to a non-phosphorylated or phosphorylated peptide lacking an Asn Pro Xaa Tyr (NPXY) sequence, and the Shc PTB domain was found

to bind to a non-phosphorylated Asn Pro Xaa His (NPXH) peptide.

Sequence conservation in the mDab1 PTB is too low to predict whether it binds phosphoinositides, but it appears, as detailed herein, to bind to phosphotyrosine containing proteins of 200, 120, 50-65 and 40 kDa from extracts of embryonic mouse heads. Since the mDab1 PTB domain is the most highly conserved part of the protein, the identification of the ligands may be central to understanding mDab1 function.

Using the mDab1 PTB domain as the "bait" in a yeast-two hybrid screen, a brain cDNA-LexA fusion library was screened for proteins that bind the mDab1 PTB. A comprehensive screen for mDab1 PTB protein ligands showed that Tyr or Phe Xaa Asn Pro Xaa Tyr (Y/FXNPXY) sequences found in the amyloid precursor protein (APP), its relatives ALP1 and ALP2, the LDL receptor related protein (LRP)/ α 2 macroglobulin receptor and Ship are high affinity ligands for the mDab1 PTB domain. APP, ALP1, ALP2, and LRP are all expressed in the developing embryonic brain, when mDab1 function is important.

The beta amyloid precursor protein (APP) is expressed in five spliced forms, all of which are transmembrane proteins. All of the splice forms have a C-terminal tail. The shortest major isoform of 165 amino acids is expressed almost exclusively in neurons and the other two major forms of 770 and 751 amino acids are expressed in both neural and non-neural cells. Abnormal cleavage of APP results in the production of small peptides that lead to Alzheimer's disease (for review, see Zheng et al., Cell 81:525-531 (1995) and Selkoe, J. Biol. Chem. 271:18295-18298 (1996); which are incorporated herein by reference). The major constituent of Alzheimer's plaques is a 38-43 amino acid peptide (amyloid β -protein ($A\beta$)). APP is transported to the cell surface where it is either cleaved by proteolysis or endocytosed. Endocytosis of the uncleaved APP molecules is mediated by the NPXY signal sequence in the cytoplasmic tail. The endocytosis of APP is the principal path for the generation of the 38-43 amino acid peptide that is subsequently secreted and deposited in the amyloid plaques. The binding of mDab1 PTB to APP may

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mice. In the reeler mouse some malpositioned neurons connect successfully (Goffinet, Anat. Embryol Berl. 157:205-216 (1979)) but Reelin regulates neuronal connectivity in some systems (Del Rio et al. Nature 385:70-74 (1997)).

5 The requirement for Dab is unveiled under conditions where dAbl tyrosine kinase activity is absent. Thus, Dab must be functional under conditions where it is not tyrosine phosphorylated by dAbl. *Drosophila* Src (dSrc) may phosphorylate Dab under these conditions. Over-expression of
10 kinase-defective dSrc during embryogenesis interferes with longitudinal connections in the CNS (Kussick et al., Oncogene 8:2791-2803 (1993)), reminiscent of *dAbl dab* double mutants (Gertler, *ibid.* (1989)). The detection of mammalian mDab1, its ability to be phosphorylated on tyrosine and then bind
15 SH2-containing PTKs, such as Src and Abl; and the binding of its PTB to tyrosine phosphorylated proteins in embryonic extracts, suggest that mDab1 might be regulated by PTKs during mammalian neurogenesis. Identification of mDab1 binding
20 partners and a receptor for Reelin will help further elucidate the underlying mechanisms by which the activities of these gene products coordinate neuronal migration and axonal guidance.

 During mammalian brain development, immature neurons migrate radially from the neuroectoderm to defined locations
25 where they adopt distinct fates, giving rise to the characteristic layered brain (Hatten et al., Curr. Opin. Neurobiol. 3:38-44, (1993) and McConnell et al., Neuron 15:761-768 (1995)). During neuronal differentiation of P19 embryonal carcinoma cells, expression of mDab1 p60, p80 and
30 p120 is induced, and the proteins are first tyrosine phosphorylated prior to neurite extension. Tyrosine phosphorylation of p80 correlates with axonogenesis becoming maximal at day 5 (E5). The expression pattern and phosphorylation of mDab1 are also regulated during mouse
35 embryonic development. At embryonic day 10.5 (E10.5), mDab1 expression was detected only in developing nerves, but at E13 additional expression was observed in developing bone, possibly in precursors to osteoclasts. mDab1 p120 is

expressed maximally at E9 and E10 and then declines, while mDab1 p80 persists in adults. Tyrosine phosphorylation of both these forms of mDab1 was observed in embryos but not in adults. mDab1 is therefore a substrate of a kinase that is active during neural development. The present invention further demonstrates that disruption of the *mammalian disabled1 (mdab1)* gene disturbs neuronal layering in the cerebral cortex, hippocampus and cerebellum. Thus mDab1 is required for correct positioning of neurons within the layered structures of the brain.

The *mdab1* mutant phenotype closely resembles that of the *reeler* mouse (Goffinet et al., Brain Res. 318:263-276 (1984); Caviness et al., J. Comp. Neurol. 147:235-254 (1973); Stanfield and Cowan, J. Comp. Neurol. 185:393-422 (1979)), in which the secreted protein Reelin fails to be produced (D'Arcangelo et al., Nature 374:719-723 (1995); Ogawa et al., Neuron 14:899-912 (1995); Hirotsune et al., Nat. Genet. 10:77-83 (1995)). In both *mdab1* and *reeler* mutant mice, neurons of a specific birthdate are found in an abnormal location. The Reelin protein, which has been proposed to act as extracellular signpost for migrating neurons, is localized normally in mice lacking mDab1 p80. Because mDab1 p80 is expressed in wild-type cortical neurons, the present invention indicates that it is part of a Reelin-regulated pathway that controls the final positioning of neurons.

mDab1 p80 is a docking protein with no known catalytic activity, so its function may be to link proteins together through its amino-terminal PI/PTB domain and tyrosine-phosphorylated motifs. This function may be regulated by extracellular signals. Thus, proteins that modify and bind to mDab1 p80, including non-receptor tyrosine kinases such as Src and Abl, may regulate neuronal migration. Mutations in non-redundant genes encoding other components of the signaling pathway might be expected to cause a *mdab1-1* - like phenotype. Mutations in *src* and *abl* do not affect brain development (Soriano et al., *ibid.* (1991); Schwartzberg et al., Cell 65:1165-1175 (1991); Tybulewicz et al., *ibid.* (1991)), but these tyrosine kinases may be redundant. An *mdab1-1*-like

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In further aspects of the invention, mDab1 proteins, peptides, fusion proteins and antibodies are used in a variety of screening and diagnostic methods. As will be evident to the common practitioner, the polynucleotide molecules, protein, peptides and antibodies of the present invention are useful in *in vitro* assays to screen for compounds capable of modulating the activity or expression of mDab1. Within these methods, the *mdab1* genes and mDab1 proteins and peptides disclosed herein are useful for generating, isolating, and characterizing endogenous and exogenous factors, drugs and other agents that can be employed in methods to evaluate and/or regulate processes involved in normal and abnormal cell migration. The general methods of the invention provide methods directed toward the diagnosis and treatment of injury and disease conditions such as metastatic cancer, reactive gliosis, neurodegenerative diseases and Alzheimer's Disease. Within such assays, test compounds may be assessed for their ability to increase or decrease mDab1 activity or expression

In further aspects of the invention, mDab1 proteins, peptides, fusion proteins and antibodies are used in a variety of screening and diagnostic methods. As will be evident to the common practitioner, the polynucleotide molecules, protein, peptides and antibodies of the present invention are useful in *in vitro* assays to screen for compounds capable of modulating the activity or expression of mDab1. Within these methods, the *mdab1* genes and mDab1 proteins and peptides disclosed herein are useful for generating, isolating, and characterizing endogenous and exogenous factors, drugs and other agents that can be employed in methods to evaluate and/or regulate processes involved in normal and abnormal cell migration. The general methods of the invention provide methods directed toward the diagnosis and treatment of injury and disease conditions such as metastatic cancer, reactive gliosis, neurodegenerative diseases and Alzheimer's Disease. Within such assays, test compounds may be assessed for their ability to increase or decrease mDab1 activity or expression

Differences that are detected and/or quantified between mDab1 expression or activity between normal and test cell populations or tissues may be diagnostic of particular

The same steps and compositions that are employed within diagnostic methods of the invention are readily adapted for use within powerful screening methods provided by the invention. Screening methods that are particularly useful within the invention include high throughput screening assays designed to identify modulators of mDab1 expression or activity. In preferred screening assays, labeled mDab1 proteins, peptides, or anti-mDab1 antibodies are employed in a similar manner as described above to detect and/or quantify expression or activity of mDab1 in comparable test and control samples. Useful control samples in this context generally include a variety of *in vivo* or *in vitro* assay mixtures suitable for detecting and/or quantifying mDab1 binding to a selected binding partner, for example Abl. Useful test samples within these screening methods contain an added test substance, i.e., a putative mDab1 modulating agent, in qualitatively or quantitatively comparable assay mixtures to those of the control samples. In screens aimed at detecting modulators of mDab1 binding to a selected binding partner, the test sample contains suitable amounts of mDab1 protein and a selected binding partner under conditions that permit the formation of mDab1-binding partner complexes in the absence of

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illustration, not limitation.

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Src interacting proteins involved in mouse embryonic development were identified using a modified yeast two-hybrid system as described by Vojtek et al. (Cell 74:205-214 (1993);

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Fumagalli et al., Nature 368:871-874 (1994)) were identified in addition to a number of cDNAs encoding novel proteins. Two identical cDNA clones, designated B3 and C46, had significant homology with the *Drosophila dab* gene and were analyzed further.

Example II

Identification and Analysis of mDAB1 cDNA

Complete cDNA clones for mDab1 were isolated by screening embryonic mouse libraries using the cDNA clone B3 as a probe (corresponding to nucleotides 579 to 1091 of SEQ ID NO:2) according to standard techniques (Sambrook et al., Molecular Cloning, 2nd ed., vol. 1-3, Cold Spring Harbor Laboratory Press, Cold Spring Harbor (1989); which is incorporated herein by reference). Three clones, designated mDab555, mDab271 and mDab217, were identified. The mDab555 and mDab271 full length cDNAs were obtained from a pCDNAI (Invitrogen, Carlsbad, CA) library of E15-17 mouse brain cDNAs (obtained from Visha Dixit, University of Michigan, Ann Arbor, MI) and mDab217 was from a λYES (Stratagene Cloning Systems) library made with embryonic stem cell cDNAs (from Zhi Chen, University of Michigan, Ann Arbor, MI).

Each cDNA was subcloned into pBLUESCRIPT (Stratagene Cloning Systems). Nested deletion mutants were generated with sequential exonuclease III and S1 nuclease treatments at 37°C (Sambrook et al., *ibid.* (1989)). Automated DNA sequencing was performed with plasmid templates on a BIOSEQUENCER (The Perkin-Elmer, Corp. - PE Applied Biosystems Division, Foster City, CA), and overlapping sequences for each clone were obtained for both strands.

The three clones represented at least three different mRNAs, encoding mDab1 isoforms with of 555, 217 and 271 residues. The nucleotide sequences and predicted amino acid sequences of the three clones mDab555, mDab217 and mDab271 are shown in SEQ ID NO:2 and SEQ ID NO:3; SEQ ID NO:4 and SEQ ID NO:5; and SEQ ID NO:6 and SEQ ID NO:7, respectively. A comparison of the sequences shows that the mDab217 mRNA diverges from mDab555 at a consensus splice donor

sequence at codon 199, encodes a further 18 residues before a termination codon, and terminates with a 3' untranslated sequence distinct from mDab555. The mDab271 mRNA contains an additional exon of 270 nucleotides inserted between codons 241 and 242 of mDab555. This exon encodes 30 residues before a stop codon. A fragment of a potential fourth cDNA was identified using RT PCR, and contained an insert in the mDab555 mRNA at another consensus splice donor sequence between residues 239 and 242. The nucleotide sequence and deduced amino acid sequence of the exon are shown in SEQ ID NO:8 and SEQ ID NO:9.

The B3 and C46 clones isolated in the two-hybrid screen include residues 106 to 274 of mDab555 (SEQ ID NO:3). The common mDab1 initiation codon is preceded by an in-frame termination codon and is in a good consensus for translational initiation (Kozak, J. Biol. Chem. 266:19867-19870 (1991)).

The chromosomal location of the *mdab1* gene was mapped using Southern blotting to follow polymorphic restriction fragments, in the progeny of the backcross (C57BL/6J X *Mus spretus*)F1 X C57BL/6J, and the reciprocal backcross. *mdab1* was localized to mouse chromosome 4, at offset 70.6. This portion of the mouse chromosome is syntenic with the human chromosome 1p32-31 region.

A database search using the mDab1 sequences identified several mDab1 relatives including p96 (Xu et al., J. Biol. Chem. 270:14184-14191 (1995), now referred to as mDab2; Genbank accession U18869) and its human homolog, DOC2 (Mok et al., Gyn. Oncol. 52:247-252 (1994); Albertsen et al., Genomics 33:207-213 (1996), Genbank Accession No. U39050), that are widely expressed proteins. A mDab-related gene, M110.5, was also identified in the *C. elegans* genome sequencing project (Wilson et al., Nature 368:32-38 (1994)). Alignments of the PTB domains of these proteins with the mDab PTB domain were calculated with GCG Pileup and similar amino acids were boxed with EGCG Prettyplot (threshold 0.8, plurality 3).

An alignment of these proteins with mDab1 and *Drosophila* Dab shows greatest sequence conservation in an

amino-terminal region of 136 residues contained within the amino acid sequence from residue 27 to 170 of SEQ ID NO:3 (Howell et al., *ibid.* (1997)); which is incorporated by reference herein). Through this region Dab and mDab1 are 52% identical (72% similar). The mDab1 and the *Drosophila* Dab proteins are related in two other areas. One stretch of 73 amino acids from residue 400 in mDab555 and 1913 in *Drosophila* Dab, is 20.5% identical (41% similar) and has an unusually high concentration of hydroxy-amino acids (19%), glutamine (12%), and proline (12%). Another stretch of 33 amino acids, from residue 508 in mDab555 and 2082 in Dab, is 36% identical (45% similar) and also rich in hydroxy amino acids (19%). The two similar hydroxy amino acid rich regions may represent conserved sites of phosphorylation. The *Drosophila* Dab protein is known to be phosphorylated to high stoichiometry on serine (Gertler, *ibid.* (1992)), which may be important for its function.

Bork & Margolis (*Cell* 80:693-694 (1995)) pointed out that the amino-terminal conserved region of Dab is distantly related in sequence to the ShcA PTB domain (also known as PI domain) (Kavanaugh & Williams, *Science* 266:1862-1865 (1994)). The ShcA PTB is composed of a β sandwich that is capped with a charged α helix (Zhou et al., *Nature* 378:584-592 (1995)), and the phosphopeptide ligand fits into a groove between them. The N-terminal part of the phosphopeptide extends as a beta strand that fits the groove and extends the beta sheet of the PTB domain, making the side chains and backbone contacts with the PTB domain. A specific phosphopeptide binds antiparallel to the β 5 strand of the PTB and is stabilized by contacts with several residues in the PTB. The phosphotyrosine on the peptide interacts with several hydrophilic and positively-charged residues at one end of the PTB, including Arg 67, Ser 151, Lys 169, and Arg 175. These residues are conserved across the Shc group of PTBs (Kavanaugh & Williams, *ibid.* (1994); Lai et al., *Mol. Cell. Biol.* 15:4810-4818 (1995); O'Bryan et al., *Proc. Natl. Acad. Sci. USA* 93:2729-2734 (1996)). Mutation of Arg 67 or Arg 175 to Gln reduced binding of the ShcA PTB to phosphorylated targets by 36% and 100%,

respectively (Zhou et al., *ibid.* (1995)). The residues amino-terminal to the phosphorylated tyrosine in the phosphopeptide bound to the ShcA PTB include the Asn Pro Xaa pTyr (NPXpY) consensus sequence (Batzer et al., *Mol. Cell. Biol.* 15:4403-4409 (1995); Kavanaugh et al., *Science* 268:1177-1179 (1995); Songyang et al., *J. Biol. Chem.* 270:14863-14866 (1995); van der Geer et al., *Curr. Biol.* 5:404-412 (1995)). These residues contact the β sheet and $\alpha 3$.

Alignment of the mDab1 and ShcA PTBs was facilitated by the solution structure of the ShcA PTB (Zhou et al., *ibid.* (1995)) and secondary structure predictions for mDab1 and the dNumb PTB (Zhou et al., *ibid.* (1995)), which represents a phylogenetic intermediate between the Shc and Dab family PTBs. mDab1 has residues that correspond with critical amino acids in the ShcA PTB that contact the phosphate moiety, including Arg 67, Ser 151 and Lys 169. However, the region between ShcA $\beta 1'$ and $\beta 2'$ is quite divergent in mDab1, and there is no apparent homolog of Arg 175 of ShcA. The PTB of IRS-1 is also divergent in this region (Eck et al., *Cell* 85:695-705 (1996); Zhou et al., *Nature Struct. Biol.* 3:388-393 (1996)). Some of the residues that contact the peptide ligand amino-terminal to the phosphotyrosine are conserved. In particular, Phe 198 in $\beta 3$ of ShcA is conserved in Dab family members and dNumb. This residue contacts the side chain of Asn -3 of the ligand. These sequence similarities suggest that the amino-terminal region of mDab1 may adopt a similar fold to the ShcA PTB and may function to bind to phosphorylated proteins or peptides.

Example III

Preparation of GST fusion protein and antibodies

Two GST fusion constructs were made with the mDab555 cDNA to facilitate the preparation antibodies. Both were cloned between the BamHI and EcoRI sites in the glutathione S-transferase gene fusion vector, pGEX-2T (Amersham Pharmacia Biotech, Inc., Piscataway, NJ) polylinker. The first construct corresponds to the region cloned in the yeast two-hybrid screen comprised of residues 107 to 243, that was PCR amplified with the oligonucleotide pairs

The Src polyclonal sera 3060 was raised to a peptide corresponding to residues 519 to 533 of c-Src (Cooper et al., Science 231:1431-1434 (1986)). The anti-phosphotyrosine monoclonal 4G10 was obtained from Deborah Morrison (National

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Expression of mDab1

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Northern analysis of the adult mouse tissues showed that mDab1 expression was largely restricted to brain. Expression was also high in E10.5 (10.5 days post coitus) embryos. Three transcripts of 5.5, 4.0 and 1.8 kb were detected. The 1.8 kb mRNA probably encodes the mDab1 217 isoform. The 5.5 and 4.0 transcripts are larger than the largest cDNA identified herein suggesting that mDab1 has an extensive 5' untranslated region or that additional spliced forms exist.

In a survey of cultured cells, mDab1 expression was found to be limited to differentiated P19 embryonal carcinoma cell cultures and various hematopoietic cell lines. mDab1 expression was not detected in the neuroblastoma- or neural crest-derived lines SY5Y and PC12, or in the fibroblast lines Rat1, 10T1/2 or NIH3T3. P19 cells are pluripotent and can be induced to differentiate into neural ectoderm when grown in

aggregates in the presence of all-trans-retinoic acid (RA) (McBurney et al., Nature 299:165-167 (1982); Jones-Villeneuve et al., Mol. Cell. Biol. 3:2271-2279 (1983)). Three to 5 days after addition of RA, P19 cultures are composed of glioblasts and neuroblasts. By 7 days, greater than 50% of the cells are axon-bearing embryonic neurons, and the remainder are glia (Rudnicki & McBurney, *ibid.* (1987)).

Expression and tyrosine phosphorylation of the mDab1 isoforms during P19 cell differentiation and neural development were determined by inducing P19 EC cells to differentiate along the neural lineage by treatment with RA at specific intervals and immunoprecipitating cell lysates with either anti-mDab(B3) or preimmune antibodies. P19 EC cells (obtained from John C. Bell and Ninan Abraham, University of Ottawa, Ottawa, Ontario, Canada) were grown and induced to differentiate as described by Rudnicki & McBurney (Teratocarcinomas and Embryonic Stem Cells: A Practical Approach, IRL Press Limited, Oxford, England, pages 19-47, (1987); which is incorporated herein by reference).

Cell lysates were prepared by lysing 1×10^6 cells on ice in 1 ml of TX-IPB (0.1 M NaCl, 1% TRITON X-100, 10 mM HEPES (N-2-hydroxyethyl-piperazine-N'-2-ethanesulfonic acid) pH 7.4, 2 mM EDTA, 0.1% 2-mercaptoethanol, 20 μ g of aprotinin per ml, 50 mM NaF, 0.2 mM NaOH, 2 mM PMSF, 1 mM phenylarsine oxide) for 10 minutes on ice. The lysates were clarified by centrifuging at 20,000 x g for 30 minutes and cleared with SEPHAROSE CL-4B (cross-linked beaded agarose at a concentration of approximately 6% agarose) (Sigma). The protein concentration was adjusted to 3 mg of protein per ml unless otherwise stated.

Cell lysates were immunoprecipitated with anti-mDab(B3) or preimmune antibodies, which were prebound and chemically crosslinked to protein SEPHAROSE beads by treatment with dimethyl pimelimidate (Schneider et al., J. Biol. Chem. 257:10766-10769 (1982); which is incorporated by reference herein). The bound antibodies were mixed with the lysates for 90 minutes at 4°C, followed by 4 washes with TX-IPB buffer.

The proteins were eluted by addition of two-times concentrated gel loading buffer (4% sodium dodecyl sulfate (SDS), 40% glycerol, 0.2M Tris-HCl (pH 6.8), 5.6 M 2-mercaptoethanol, 5 mM EDTA, 0.02% bromophenol blue). The samples were boiled for 10 minutes prior to analysis by SDS polyacrylamide electrophoresis (SDS PAGE). The immunoprecipitates were electrophoresed by SDS PAGE and were immunoblotted to detect mDab1 with anti-mDab(B3) antibody and phosphotyrosine with 4G10 antibody. Immunoblotting was performed as described by Howell & Cooper (ibid. (1994); which is incorporated by reference herein). Immunoreactive proteins were visualized by enhanced chemiluminescence (ECL) detection system (Amersham Pharmacia Biotech Inc.).

Differentiated P19 cultures were found to express the 60, 80 and 120 kDa mDab1 isoforms by immunoblot analysis of anti-mDab1 immunoprecipitations from lysates of these cells. However, in undifferentiated P19 cells, only p80 was observed. The abundance of this isoform increased about 5 fold during neuronal differentiation, then declined after day 5. The p60 and p120 isoforms were not detected in undifferentiated cells, and their expression peaked at day 3 of differentiation.

Immunoblot analysis of the same immunoprecipitates with anti-phosphotyrosine antibodies revealed that the p60, p80 and p120 were tyrosine phosphorylated during differentiation, with the maximal phosphorylation of all proteins occurring at day 5. No change in mDab1 expression or tyrosine phosphorylation was detected when P19 cells were induced to differentiate into muscle lineages (Edwards et al., Mol. Cell. Biol. 3:2280-2286 (1983)). Various isoforms were detected in the hematopoietic cell lines LSTRA (120, 36 kDa), Jurkat (120, 36 kDa), K562 (36 kDa), and 32D (120, 45 kDa).

Using anti-peptide antibodies, it was found that the p80 and p60 forms of mDab1 contain the common amino-terminal sequence encoded by all of the cloned cDNAs, and that the p120 and p80 forms contain the C-terminal sequence specific to the mDab555 mRNA. In vitro translated mDab555 has an apparent molecular mass of 75 kDa. When expressed in fibroblasts,

mDab1 tyrosine phosphorylation was examined at E13 in mice homozygous for mutations in the *src*, *fyn* or *abl* genes (Soriano et al., Cell 64:693-702 (1991); Tybulewicz et al., (Cell 65:1153-1163 1991); Stein et al., Cell 70:741-750 (1992)) to determine whether the Src, Fyn or Abl kinases phosphorylate mDab1 during mouse brain development. mDab1 was

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together. All secondary, tertiary, antibody alone, and mDab1 preimmune controls were negative. Nerve tracts were identified by double label immunostaining with antibody to a general axonal marker as described by Placzek et al. (ibid. (1990)). mDab1 expression was observed in the head in neural tracts corresponding to the developing cranial nerves, such as the oculomotor and the trochlear nerves. In the body, mDab1 expression in the spinal accessory nerve and dorsal root ganglia was apparent. At day E13, mDab1 expression was observed in sensory nerves that innervate the vibrissae, and in developing bone in the extremities. All nerves identified at these times by neurofilament antibody also expressed mDab.

These results demonstrate that mDab1 is localized in nerves and is tyrosine phosphorylated at times when the nervous system is undergoing rapid expansion and axonal networks are developing. mDab1 is expressed in the adult brain, but is not detectably tyrosine phosphorylated suggesting that mDab1 interacts with protein-tyrosine kinases during the development of the nervous system and may act to transduce signals during development.

f. Interaction of mDab1 with Src

The identification of mDab1 in a yeast two-hybrid screen using Src as the bait suggested that mDab1 would interact with phosphotyrosine kinases (PTKs). To determine the nature of the interaction between mDab1 and Src, β -galactosidase expression in yeast expressing mDab1 clone B3 and various Src mutant-LexA fusions including Src(FF), Src(Δ SH3), Src(295R) and Src(SH2').

The Src wild type cDNA and each mutant were amplified by polymerase chain reaction (PCR) amplification using the primers (5'-CTCGGATCCTCATGGGGAGCAGCAAGAGCA-3') (SEQ ID NO:16) and (5'-CTCATGCATCCTATAGGTTCTCTCCAGG-3') (SEQ ID NO:17), directed to the amino- and carboxyl-terminus of Src, respectively. Each PCR product was digested with BamHI and NsiI and ligated into the BamHI and PstI cloning sites of the pBTM116 vector (Vojtek et al., ibid. (1993) and Hollenberg et al., ibid. (1995)) to generate the Src-LexA fusion constructs.

Pairs of Src-LexA and mDab1-VP16 fusion proteins were coexpressed in *Saccharomyces cerevisiae* strain L40 as described previously. β -galactosidase activity was detected by filter assay. The Src mutants Src(Δ SH3) and Src(FF) interacted with mDab1 as strongly as did wild-type Src. However, neither the Src(SH2') nor the Src(295R) mutants interacted with mDab1. The Src-mDab1 interaction therefore requires tyrosine phosphorylation of mDab1 but not Src and requires the SH2 domain and not the SH3 domain. This is consistent with the model that phosphorylation of mDab1 in the region encoded by the cDNA clone B3 provides a binding site for the SH2 domain of Src.

Example V

Interaction of mDab1 and Src and other phosphotyrosine kinases in mammalian cells

a. Interaction of mDab1 with Src

To test whether the mD ab555 product, p80, was phosphorylated by Src in mammalian cells, mDab555 (p80) was expressed alone or together with activated Src (Src527F) in 293T fibroblasts. For expression in mammalian cells the entire open reading frame of the mDab555 cDNA was PCR amplified with the oligonucleotides 5'-CGCGGATCCAGGATGTCAACTGAGACA-3' (SEQ ID NO:18) and 5'-CGCGGATCCTTCACTGGGCGACTGTGAGT-3' (SEQ ID NO:19) and ligated into the BamHI site of the pLXSH retroviral vector (Miller et al., Meth. Enzymol. 217:581-599 (1993)). The retroviral vector pLXSHD-Src(527F) (Howell & Cooper, Mol. Cell. Biol. 14:3813-3822 (1994); which is incorporated by reference herein) contained the activated Src527F cDNA in the pLXSH retroviral vector.

Virus was produced (Afar et al., Science 264:424-426 (1994)) by cotransfecting the retroviral DNAs with a ecotropic packaging vector containing gag, pol and env genes obtained from Owen Witte (University of California, Los Angeles, CA) into 293T cells (obtained from Robert Eisenman,

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immunoprecipitations with 5 μ g of GST fusion protein immobilized on glutathione agarose beads. Phenyl phosphate and phosphoserine were used at 50 mM for competition experiments. After washing, mDab1 bound to the immobilized SH2 domains was detected by immunoblotting. Tyrosine phosphorylated but not control mDab1 associated with the Src, and Fyn SH2 domains *in vitro*. mDab1 also interacted with the Abl SH2 domain, but less well than with Src or Fyn, and did not interact with the Csk SH2 domain. The Abl and Csk SH2 domains do form high affinity complexes with other tyrosine phosphorylated molecules however (Sabe et al., Proc. Natl. Acad. Sci. USA 91:3984-3988 (1994); Duyster et al., *ibid.* (1995)).

c. Overexpression of mDab1 and Src527F in mammalian cells

To examine whether mDab1 and Src527F would form complexes in mammalian cells, both proteins were overexpressed in Rat-1 fibroblasts (obtained from Robert Eisenman, Fred Hutchinson Cancer Research Center, Seattle, WA). Cell lysates were immunoprecipitated with either anti-Src antibodies or preimmune serum, and mDab1 was detected by immunoblotting with anti-mDab1 antibody essentially as described above. mDab1 co-immunoprecipitated with Src, and was detected with both anti-mDab1 antibodies and antiphosphotyrosine antibodies. Approximately 1% of the phosphorylated mDab1 that was present in the total cell lysate was immunoprecipitated. In addition Src was detected in anti-mDab immunoprecipitates. A 60 kDa tyrosine-phosphorylated protein detected in mDab1 immunoprecipitates from 293T cells expressing mDab1 and Src527F was confirmed to be Src. These results demonstrated that Src and mDab1 p80 formed complexes stable enough to be isolated from cells in the presence of non-ionic detergent.

d. Identification of Tyrosine residues involved in Src SH2 binding

To test the effect of mutations at the Tyr 198 and Tyr 200 residues on binding sites for the Src SH2 domain, a mutant mDab555 was generated which contained Phe residues in place of both Tyr 198 and Tyr 200. The mutant mDab555 was generated by oligonucleotide site-directed mutagenesis as described previously (Kunkel et al., Meth. Enzymol. 154:367-382 (1987); which is incorporated herein by reference), with the oligonucleotide 5'-CACAACTGAAGTGGGACGGGATCTTCCAC-3' (SEQ ID NO:23). This mutagenesis simultaneously introduced both mutations and was designated mDab198/200F. Mutants were identified by screening colonies for the introduction of a unique BbsI site into the mDab555 cDNA and were confirmed by sequence analysis. The mutant cDNA was inserted into the retroviral vector as described above to analyze the effect of these mutations on binding sites for the SH2 domain of Src or other PTKs. Lysates from 293T cells transfected with retroviral vectors encoding mDab555 alone; Src 527F alone; or Src527F mDab198/200F were either bound to immobilized GST fusions proteins with the Src, Csk, Fyn or Abl SH2 domain or were immunoprecipitated with anti-mDab1 or preimmune antibodies essentially as described above. After washing,

mDab1 bound to the immobilized SH2 domains was detected by immunoblotting. The *in vivo* association between mDab1 and Src was reduced about 2-fold by the mutation. These results suggest that Tyr 198, or Tyr 200 and another of the six tyrosines in the B3 region of mDab1 may be Src binding sites.

e. The association between Src and mDab1 in differentiating P19 cells

To determine whether the interaction between mDab1 and Src could be detected under conditions where neither of the proteins was overexpressed, Src immunoprecipitates were prepared from lysates of differentiating P19 cells and associated proteins were detected by phosphorylation with [32 P]ATP *in vitro*. Lysates from P19 cells were induced to differentiate with retinoic acid for three, five or seven days. The lysates were immunoprecipitated as described above with anti-Src or preimmune antibodies and incubated with [γ - 32 P]ATP to allow phosphorylation. Immunoprecipitation was carried out as described above, with the exception that after the four washes with TX-IXB, a further two washes were done with PAN buffer (100 mM NaCl, 10 mM PIPES (piperazine-N,N'-bis-2-ethanesulfonic acid) pH 7.0, 20 μ g of aprotinin per ml) prior to incubation in UKB (10 mM PIPES (pH 7.0), 10 mM MnCl₂, 0.50 μ M [γ - 32 P]ATP (3,000 Ci/mmol)) for 15 minutes at 30°C. The reactions were stopped by elution of proteins described above. Labeled proteins were eluted and reimmunoprecipitated with either anti-Src, anti-mDab1 or preimmune antibodies. Samples analyzed by re-immunoprecipitation were lyophilized to remove the 2-mercaptoethanol, and then diluted 1:50 (from the original volume) into TX-IPB and mixed with antibodies overnight at 4°C. Proteins were visualized by autoradiography. Several labeled proteins including Src and proteins of approximately 60 and 80 kDa were observed.

To test whether mDab1 proteins were present, the radiolabeled proteins were eluted from the immunoprecipitates and reimmunoprecipitated with either preimmune serum, anti-Src antibody, or anti-mDab(B3). The p60 and p80 forms of mDab1 were recovered in the second immunoprecipitation of Src kinase

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f. Association of mDab1 with tyrosine phosphorylated proteins

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to the ShcA PTB. The binding of the 55-60 kDa proteins to both the wild-type and the mutant mDab PTB, and in the presence of phosphoamino acid competitors, may suggest that the mDab PTB is also capable of protein-protein interactions independent of phosphorylation.

Example VI

Disruption of the mammalian disabled1 (mdab1) gene disturbs neuronal layering in the cerebral cortex, hippocampus and cerebellum

An *mdab1*-1 targeting vector for deletion of *mDab1* was constructed by first inserting a blunted 0.9-kb BseRI-BglI fragment corresponding to intronic sequences 5' to the exon encoding residues 23 to 69 of the *mdab1* gene in the PGKneolox2DTA vector (Soriano, Devel. 124:2691-2700 (1997); which is incorporated herein by reference). A 4-kb EcoRI-XbaI fragment from the *mdab1* gene 3' to the same exon was linked and was then ligated 3' into the SalI site between the PGKneo and PGK-DT sequences producing the targeting vector designated p80KO1. Plasmid p80KO1 was designed with the phosphoglycerate kinase (PGK) promoter driving neomycin phosphotransferase in place of 2 kb of genomic sequences that contained the first exon of the PI/PTB domain. Homologous sequences of 0.9 (5') and 4 kb (3') flank the PGKneo cassette. The targeting vector permitted nonhomologous integrants were counter-selected with the PGK-diphtheria toxin (DT) cassette.

The *mDab1* gene was disrupted by homologous recombination with p80KO1 in embryonic stem cells. AK7 embryonic stem (ES) cells (1×10^7) were electroporated with 20 μ g linearized p80KO1. Cell culture and blastocyst injections were done as described by Soriano (*ibid.* (1991); which is incorporated by reference herein). Mice heterozygous for the altered allele (*mdab1*-1) were generated by standard blastocyst manipulation and mouse breeding. Genotyping of resulting mice was confirmed PCR genotyping using oligonucleotides P1 (5'-GTCAGGCTTCCTAAGTAGAAAGGA-3') (SEQ ID NO:24), P2 (5'-TTCCAGGAGCGAAATCACTCAACC-3') (SEQ ID NO:25), and P3 (5'-GGGAAAAGCGCCTCCCCTACCCGGT-3') (SEQ ID NO:26).

Hematoxylin-eosin (H&E), Nissl, and Bielschowski staining were done following standard protocols. Coronal section of the neocortex were stained with Bielschowski stain, the hippocampus was stained with Hematoxylin-eosin and cerebellum was immunostained with anti-Calbindin antibody and counterstained with Nissl. Anti-CR 50, anti-BrdU (Becton Dickinson), and anti-calbindin immunohistochemistry was carried out as generally described (Ogawa et al., Neuron

The mutant cerebellum is small and has obviously altered structure. A normal P25 cerebellum has an outer cell-poor (molecular) layer, a single layer of Purkinje cells (PCs) with dendritic arbors extending into the molecular layer, a broad inner granule layer (IGL), and an underlying layer of white matter. This structure forms after birth. At birth, the wild-type cerebellum has PCs in a central mass and granule cells in an external granule layer (EGL). Starting at about P5, the PCs disperse to form a monolayer and the granule cells proliferate and migrate inward to form the IGL. The cerebellum of a *mdab1-1* mutant at P25 was small and unfoliated, leaving the midbrain exposed, and resembles a prenatal wild-type

cerebellum. The PCs appear to have matured normally, because they express both calbindin and zebrin II (Lannoo et al., J. Comp. Neurol. 310:215-233 (1991)), but they fail to disperse into a monolayer and their dendrites were randomly oriented. Perhaps as a result, normal PC-granule cell interactions were disrupted (Goffinet, Anat. Embryol Berl. 157: 205-216 (1979)), fewer granule cells mature, and although they migrate inward the majority remain superficial to the Purkinje cells. Where the EGL and PCs come into contact, the histology was more normal, suggesting that where PC-granule cell interaction was not seriously affected the granule cells develop more normally. Therefore the primary defect may be a failure of the PCs to disperse into a monolayer.

15 In the neocortex, the position and fate of a neuron
is strongly correlated with the neuron's birthdate (McConnell
et al., *ibid.* (1995)). Neurons born on successive days
migrate past their predecessors, further outward from the
neural tube (McConnell, Curr. Opin. Neurobiol. 2:23-27
(1992)). This has been shown by analyzing the brains of
20 adults that were labeled in utero with thymidine analogues,
such as 5-bromodeoxyuridine (BrdU). Cells undergoing their
last S phase during the labeling period retain the label in
their DNA, whereas cells that continue to cycle dilute the
label over time. Most neurons that are marked early
25 (embryonic day 11, E11) lie deep in the neocortex and
differentiate as polymorphic cells, while most neurons marked
on E16 end up in superficial layers and differentiate as small
pyramidal cells. To test whether cortical neurons migrate
correctly in *mdab1-1* mutant mice, mice were treated in utero
30 with BrdU and their brains analyzed at P25. Nuclei that were
labeled with BrdU on E11 were found deep in the cortex of
wild-type animals (layer VI) and superficial in the *mdab1-1*
mutants. Conversely neurons labeled on E16 were predominantly
in the superficial cortex (layers II-III) of wild-type mice,
35 but deep in the cortex of *mdab1-1* mutant littermates. This
showed that the final positions of cortical neurons are
abnormal in *mdab1-1* mutant mice. Because large pyramidal
cells, normally located deep in the cortex, were found near

These results indicated that *mdab1-1* mutant mice were similar to the *reeler* mutant mice (Goffine, *ibid.* (1979); Caviness et al., Brain Res. 256:293-302 (1982); Hoffarth et al., *ibid.* (1995)). In both cases, the defects seen in the cortex are likely a consequence of altered neuronal migration indicating that *mDab1* p80 and Reelin are involved in the same pathway that regulate neuronal migration. Reelin has been proposed to serve as a marker for the localization at which migrating cortical neurons come to rest (D'Arcangelo et al., Nature 374:719-723 (1995); Ogawa et al., *ibid.* (1995)). Reelin is expressed by pioneer Cajal-Retzius (CR) neurons, that are born early and occupy the outermost layer of the neocortex, immediately under the pia. p80 responds to the Reelin signal as demonstrated by an increase in *mDab1* phosphorylation in response to Reelin protein.

In the E16 cerebellum, p80 was expressed in the region where the PCs were coalescing. p80 expression is also observed in mature PCs. p80 was not detected in the EGL, and it is unlikely that Bergmann glia express p80 because these cells have cytoplasmic projections across the EGL and no

In the E16 cerebellum, p80 was expressed in the region where the PCs were coalescing. p80 expression is also observed in mature PCs. p80 was not detected in the EGL, and it is unlikely that Bergmann glia express p80 because these cells have cytoplasmic projections across the EGL and no

fluorescence was detected there. Because PCs in *mdab1-1* mutants are malpositioned at P0 prior to the granule cell ingress, the primary defect in the mutant cerebellum may be due to defects in the PCs, and defects in granule cell number and position might be secondary. It has been shown previously that granule cells depend upon adjacent PCs for trophic support (Smeyne et al., Mol. Cell. Neurosci. 6:230-251 (1995)), and that granule cells make Reelin (Miyata et al., J. Comp. Neurol. 372:215-228 (1996)). Because Reelin expression was not altered in the *mdab1-1* mutants and because mDab1 p80 was expressed by the affected cell types in the neocortex and cerebellum, it seems likely that mDab1 p80 acts cell-autonomously.

Example VII

Comprehensive screens for mDab1 PTB domain protein ligands

To identify tyrosine-phosphorylated ligands for the mDab1 PTB domain a modified yeast two hybrid system was used to screen brain and hematopoietic cell cDNA libraries. The modified two hybrid system utilized a yeast strain that expressed the protein tyrosine kinase Src essentially as described by Keegan and Cooper (Oncogene 12:1537-1544 (1996)), Lioubin et al. (Genes & Devel. 10:1084-1095 (1996)) and PCT/US96/14754, each of which are incorporated by reference herein. The mDab1 PTB domain was expressed as a fusion protein with the LexA DNA binding domain, and brain and hematopoietic cell cDNA libraries were expressed as fusion proteins with a transcriptional activator domain. Interaction between mDab1 and the transcriptional activator fusion protein was assessed using two reporter genes that carry the LexA operator sequence, *HIS3* and *lacZ* essentially as described above.

Transformation with the brain and hematopoietic libraries gave 3×10^6 and 0.3×10^6 yeast transformants, respectively. Of these, 72 clones were identified that expressed the *HIS3* and *lacZ* genes in the presence of the LexA-mDab1 PTB fusion protein but not in the presence of a control

The ability of the mDab1 PTB domain to interact directly with synthetic peptides based on the sequences identified in the two-hybrid screen was tested to identify the optimal sequence for mDab1 PTB domain binding. A GST-mDab1 PTB domain fusion protein was purified and radioactively-labeled by phosphorylation with protein kinase A and radioactive ATP. The purified, radioactive fusion protein was then incubated with a sheet of cellulose paper onto which different 15 to 17 residue synthetic peptides had been synthesized in a grid array (Niebuhr, et al., EMBO J. 16:5433-5444 (1997), incorporated herein by reference). Each sheet contained up to 100 different peptide sequences. After incubation, the sheet was washed and exposed to film, and bound PTB domain fusion protein was quantified. Auto-radiography of the filter after a binding reaction with a radioactively labeled GST-PTB domain fusion protein shows that differences in peptide sequence influences the amount of PTB bound. The mDab1 PTB domain was tested against peptides containing NPXY motifs from APP, the APP relatives APLP1 and APLP2, LRP, the LRP-related LDL receptor, Ship, and known ligands for the Shc and IRS-1 PTB domains, namely the EGF receptor, HER3 receptor, NGF receptor (TrkA), and insulin

To determine which residues in the APP sequence are involved in the interaction with the mDab1 PTB, an array of peptides based on the APP sequence with alanine substituted at each position in turn were synthesized. The ability of each peptide to bind to the PTB domain was compared to wild-type. The standard deviation of the procedure was shown to be

To test whether mDab1 would bind to the cytoplasmic tail of the APP protein, extracts of P19 cells were incubated with GST fusion proteins and subjected to immunoblot analysis as described above. Briefly, extracts of P19 cells, which

The affinity of the interaction between the mDab1 PTB domain and the APP synthetic peptide was determined by fluorescence depolarization (Li et al., Proc. Natl. Acad. Sci. USA, 74:7204-7209 (1997) incorporated herein by reference). When a fluorescently-labeled peptide is excited with polarized light, it emits fluorescence that is partially depolarized. The extent of depolarization depends on the rotational diffusion of the fluor. When bound to a large protein, such as a PTB domain, the rate of tumbling is reduced, and the emitted light retains greater polarization. The amount of residual fluorescence polarization is thus directly proportional to the percentage of fluorescent peptide that is bound to the protein. When trace amounts (approximately 1 nM) of fluorescein-labeled APP peptide was incubated with

5

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Determination of mDab1 phosphotyrosine binding domain interaction with phosphoinositides

35

GST-mDab1 PTB was found to bind to LUVs containing various phosphoinositides, but was unable to bind to LUVs lacking phosphoinositide. The efficiency of binding to the various phosphoinositides was found to vary with PdtIns3,4,5P3 being less than that to PtdIns4P or PtdIns4,5P2. Binding of

mDab1 PTB to nonphosphorylated PtdIns was also found to be inefficient.

mDab1 PTB binding to LUVs containing phospho-
 inositides in the presence of various phosphorylated isomers
 5 of inositol was also examined to determine whether the binding
 of mDab1 PTB to phosphoinositides was specific or was the
 result of a strong affinity for strongly anionic
 phospholipids. GST-mDab1 PTB binding to LUVs containing
 PtdIns4,5P2 was tested in the presence of 100 μ M D-
 10 Inst1,4,5P3, L-Ins1,4,5P3 and other inositol phosphates.
 Binding was competed with D-Ins1,4,5P3, but not by L-
 Ins1,4,5P3. When tested at 30 μ M, weak competition was
 detected with D-Ins1,4,5P3 and not other inositol phosphates.
 The pattern of competition obtained suggested that the mDab1
 15 PTB domain specifically recognized the phosphorylated isositol
 headgroup present on PtdIns4,5P2. The stereospecificity
 implies that the phosphates on the phosphoinositide bind to
 specific sites on the PTB domain, and that the PTB domain does
 not bind to all highly-phosphorylated compounds. However, the
 20 concentration of inhibitor needed to reduce binding was high,
 suggesting that binding to the phosphoinositide may also be of
 low affinity.

The GST fusion proteins were also tested to
 determine if possible dimerization of the fusion protein
 25 altered to binding specificity of the PTB to multimeric
 ligands, such as an LUV containing many molecules of
 PtdIns4,5P2. The effects of dimerization were tested by
 cleaving the GST-mDab1 fusion protein with thrombin prior to
 incubating the PTB with LUV containing PtdIns4,5P2. Released
 30 GST was found not to bind to the LUVs, but cleaved mDab1 and
 uncleaved fusion protein were found to bind to the LUVs with
 similar efficiency. These results suggested that GST-mediated
 dimerization would not artificially raise the apparent
 affinity determined for the mDab1 PTB when tested as a GST
 35 fusion protein.

Although the foregoing invention has been described
 in some detail by way of illustration and example for purposes
 of clarity of understanding, it will be obvious that certain

changes and modifications may be practiced within the scope of the invention. All publications and patents mentioned in this specification are herein incorporated by reference into the specification to the same extent as if each individual
5 publication or patent was specifically and individually indicated to be incorporated herein by reference.

WHAT IS CLAIMED IS:

1 1. An isolated and purified polynucleotide
2 molecule which encodes mammalian Dab1 Disabled protein 1, or a
3 fragment thereof, wherein the mammalian Disabled protein
4 comprises a phosphotyrosine binding domain and is capable of
5 associating with Src, Abl or Fyn, or a complementary sequence
6 thereof of claim 1.

1 2. The polynucleotide of claim 1, which is genomic
2 DNA, or a cDNA sequence.

1 3. The polynucleotide of claim 1, which codes for
2 murine Disabled protein 1 (mDab1).

1 4. The polynucleotide of claim 1, which encodes a
2 polypeptide sequence as depicted in SEQ ID NO:3, SEQ ID NO:5,
3 or SEQ ID NO:7.

4
5 5. The polynucleotide of claim 1, which hybridizes
6 to an oligonucleotide of 25 or more contiguous nucleotides of
7 SEQ ID NO:2, SEQ ID NO:4 or SEQ ID NO:6, or a complement of
8 said nucleotide sequence, and which codes for a polypeptide
9 comprising a phosphotyrosine binding domain and is capable of
10 associating with Src, Abl or Fyn.

1 6. A probe which comprises an oligonucleotide
2 capable of specifically hybridizing with a polynucleotide
3 sequence which encodes a mammalian Disabled protein 1, or
4 allelic and species variants thereof.

1 7. The probe of claim 6, which comprises from
2 about 15 to about 60 nucleotides in length.

1 8. The probe of claim 6, which further comprises a
2 detectable signal.

1 14. The host cell of claim 13, wherein the host
2 cell is a prokaryotic or eukaryotic cell.

1 25. The antibody antisera of claim 23, wherein the
2 animal is immunized with a polypeptide comprising
3 substantially the amino acid residue sequence as depicted in
4 SEQ ID NO:14 or as substantially depicted in SEQ ID NO:15.

1 34. The method of claim 33, wherein the
2 identification and quantification of mammalian Disabled
3 protein 1 expression is evaluated by Southern blot, Northern
4 blot, or polymerase chain reaction analysis.

1 35. A method for scanning for agents capable of
2 modulating the activity of or expression of mammalian Disabled
3 protein 1, the method comprising assessing the ability of an
4 agent to be tested to modulate the expression of mammalian
5 Disabled protein or to modulate the ability of mammalian
6 Disabled protein to associate with Src, Abl or Fyn in a cell
7 sample as compared to a control sample to which the agent to
8 be tested has not been added.

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

(51) International Patent Classification ⁶ : C12N 15/09, C07K 14/47, C07H 21/04, A61K 39/395		A1	(11) International Publication Number: WO 99/09153 (43) International Publication Date: 25 February 1999 (25.02.99)
(21) International Application Number: PCT/US98/17384 (22) International Filing Date: 21 August 1998 (21.08.98) (30) Priority Data: 60/056,473 21 August 1997 (21.08.97) US (63) Related by Continuation (CON) or Continuation-in-Part (CIP) to Earlier Application US 60/056,473 (CIP) Filed on 21 August 1997 (21.08.97) (71) Applicant (for all designated States except US): FRED HUTCHINSON CANCER RESEARCH CENTER [US/US]; 11 Fairview Avenue N., MS: C2M 027, P.O. Box 19024, Seattle, WA 98109-1024 (US). (72) Inventors; and (75) Inventors/Applicants (for US only): COOPER, Jonathan, A. [GB/US]; 643 Randolph Place, Seattle, WA (US). HOWELL, Brian, W. [CA/US]; 1808 E. Thomas, Seattle, WA 98112 (US).		(74) Agents: PARMELEE, Steven, W. et al.; Townsend and Townsend and Crew LLP, 8th floor, Two Embarcadero Center, San Francisco, CA 94111 (US). (81) Designated States: AU, CA, JP, US, European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE). Published <i>With international search report.</i> <i>Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>	
(54) Title: ISOLATION AND EXPRESSION OF A DISABLED PROTEIN GENE mDab1 AND METHODS (57) Abstract <p>A mammalian homology of <i>Drosophila</i> Disabled protein has been identified and cloned. In particular, the murine homolog designated mDab1 has been cloned and expressed. mDab1, when tyrosine phosphorylated, binds to the SH2 domain of Src, Abl and Fyn. Antibodies specific for mDab1 are provided as are methods for the screening of agents for their ability to modulate mDab1 activity. Methods for diagnosing Disabled protein associated disease are also provided.</p>			



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(Includes Reference to PCT International Applications)

ATTORNEY'S SOCKET NUMBER

14538A-004010

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name.

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled:

ISOLATION AND EXPRESSION OF A DISABLED PROTEIN GENE MdaB1 AND METHODS

the specification of which (check only one item below):

☐ is attached hereto.☐ was filed as United States application

Serial No. _____

on _____

and was amended

on _____ (if applicable).

☒ was filed as PCT international applicationNumber PCT/US98/17384on 21 August 1998

and was amended under PCT Article 19

on _____ (if applicable).

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is material to the examination of this application in accordance with Title 37, Code of Federal Regulations, §1.56(a).

I hereby claim foreign priority benefits under Title 35, United States Code, §119 of any foreign application(s) for patent or inventor's certificate or of any PCT international application(s) designating at least one country other than the United States of America listed below and have also identified below any foreign application(s) for patent or inventor's certificate or any PCT international application(s) designating at least one country other than the United States of America filed by me on the same subject matter having a filing date before that of the application(s) of which priority is claimed:

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			<input type="checkbox"/> YES <input type="checkbox"/> NO
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U.S. APPLICATION NUMBER	U.S. FILING DATE	PATENTED	PENDING	ABANDONED
60/056,473	21 August 1997			X
PCT APPLICATIONS DESIGNATING THE U.S.				
PCT APPLICATION NO	PCT FILING DATE	U.S. SERIAL NUMBERS ASSIGNED (if any)		
PCT/US98/17384	21 August 1998			

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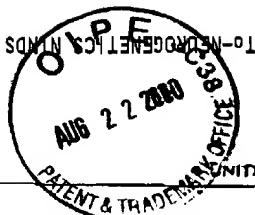
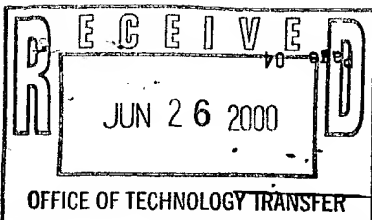
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NATURE OF INVENTOR 201	SIGNATURE OF INVENTOR 202	SIGNATURE OF INVENTOR 203
TE 5/31/00	DATE	DATE



Received June 19-00 05:24pm From 2066676349

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Combined Declaration For Patent Application and Power of Attorney (Continued)
(Includes Reference to PCT International Applications)

ATTORNEY'S DOCKET NUMBER
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U.S. APPLICATIONS		STATUS (Check one)		
U.S. APPLICATION NUMBER	U.S. FILING DATE	PATENTED	PENDING	ABANDONED
60/056,473	21 August 1997			X
PCT APPLICATIONS DESIGNATING THE U.S.				
PCT APPLICATION NO	PCT FILING DATE	U.S. SERIAL NUMBERS ASSIGNED IF ANY		
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POWER OF ATTORNEY: As a named inventor, I hereby appoint the following attorney(s) and/or agent(s) to prosecute this application and transact all business in the Patent and Trademark Office connected therewith. (List name and registration number)

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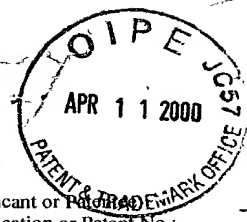
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Attorney Docket No.: 14538A004010US

**VERIFIED STATEMENT (DECLARATION) CLAIMING SMALL ENTITY STATUS
(37 CFR 1.9(f) & 1.27(d)) - NONPROFIT ORGANIZATION**

Applicant or Patent Owner:

Jonathan A. Cooper and Brian W. Howell

Application or Patent No.:

Filed or Issued:

Title:

ISOLATION AND EXPRESSION OF A DISABLED PROTEIN GENE MdaB1 AND METHODS

I hereby declare that I am an official empowered to act on behalf of the nonprofit organization identified below:

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Fred Hutchinson Cancer Research Center

Address of Nonprofit Organization:

Office of Technology Transfer

1100 Fairview Avenue N., M/S: C2M 027

P.O. Box 19024

Seattle, WA 98109-1024

Type of Nonprofit Organization:

- ☐ University or other institution of higher education.
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- ☒ the specification filed herewith;
☐ Application No. _____, filed _____;
☐ Patent No. _____, issued _____.

I hereby declare that rights under contract or law have been conveyed to and remain with the nonprofit organization regarding the above identified invention.

If the rights held by the nonprofit organization are not exclusive, each individual, concern or organization having rights in the invention is listed below* and no rights to the invention are held by any person, other than the inventor, who would not qualify as an independent inventor under 37 CFR 1.9(c) if that person made the invention, or by any concern that would not qualify as a small business concern under 37 CFR 1.9(d), or a nonprofit organization under 37 CFR 1.9(e).

*NOTE: Separate verified statements are required from each named person, concern or organization having rights to the invention averring to their status as small entities. (37 CFR 1.27)

Name:

Address:

☐ Individual ☐ Small Business Concern ☐ Nonprofit Organization

Name:

Address:

☐ Individual ☐ Small Business Concern ☐ Nonprofit Organization

I acknowledge the duty to file, in this application or patent, notification of any change in status resulting in loss of entitlement to small entity status prior to paying, or at the time of paying, the earliest of the issue fee or any maintenance fee due after the date on which status as a small entity is no longer appropriate. (37 CFR 1.28(b))

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application, any patent issuing thereon, or any patent to which this verified statement is directed.

Name of Person Signing:

Douglas J. Shaeffer

Title in Organization of Person Signing:

V.P. and General Counsel

Address of Person Signing:

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P.O. Box 19024

Seattle, WA 98109-1024

Signature

Date

January 18, 2000

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WO 99/09153

SEQUENCE LISTING

<110> Cooper, Jonathon A
Howell, Brian W
Fred Hutchinson Cancer Research Center

<120> ISOLATION AND EXPRESSION OF A DISABLED PROTEIN GENE
mDab1 AND METHODS

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